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(54) Title: METHOD FOR THE STERILIZATION OF BIOLOGICAL MATERIALS

(57) Abstract

The present invention relates to the decontamination and sterilization of pathogens, such as viruses, bacteria, and fungi present in biological materials, i.e., protein preparation, carbohydrate solutions, lipid suspensions, or cells, e.g., for administration into human or animal subjects, or for use in tissue culture systems. In particular, the invention provides a method for decontaminating a biological liquid while minimizing degradation of biological materials, such as proteins and cells. The method of the invention comprises contacting the biological liquid with sufficient reactive oxygen to deactivate microorganisms that may be present in the biological liquid by taking account of a reactive oxygen demand of the biological liquid. Critically, the maximum period of time, or effective treatment window, that the reactive oxygen is in contact with the biological liquid averts degradation of the biological material that may be present in the biological liquid. The reactive oxygen demand of the biological liquid is a function of a reactive oxygen demand of a liquid carrier and a reactive oxygen demand of any biological material that may be present in the liquid carrier. The greater the reactive oxygen demand of the biological liquid, the greater the rate of reactive oxygen transfer required to deactivate microorganisms within the effective treatment window. In specific embodiments, the invention provides for sterilization of immunoglobulin and various cells without degrading them.

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METHOD FOR THE STERILIZATION OF BIOLOGICAL MATERIALS

FIELD OF THE INVENTION

The present invention relates to the decontamination and sterilization of microorganisms, such as viruses, bacteria, and fungi, particularly pathogenic microorganisms, present in biological materials, *i.e.*, protein preparations, carbohydrate solutions, lipid suspensions, or cells, *e.g.*, for administration into human or animal subjects, or for use, *in vitro*, for example in tissue culture systems. The invention particularly relates to increasing the safety of the blood supply, and blood products.

BACKGROUND OF THE INVENTION

Oxygen is an allotropic element, with the most common form of the gas being the di-atomic form (O_2). Ozone (O_3) and singlet oxygen ($^1O_2$) are other forms of the gas that occur naturally and that can be created artificially.

Ozone is the triatomic form of oxygen and is relatively unstable. One method for generating ozone is to expose O_2 gas to electromagnetic radiation having a wave length of about 180 to 187 nm. Other methods of forming ozone are well-known. Singlet oxygen is the monatomic form of oxygen and is highly unstable. One method for generating singlet oxygen is to expose ozone to ultra-violet light at a wavelength of about 253.7 nm. It is also known to expose a mixture of oxygen gas containing ozone and singlet oxygen to visible light at a wavelength of between about 500 and 800 nm to further induce the formation of singlet oxygen. Other methods of forming singlet oxygen are well known.

Ozone, singlet oxygen, and even triplet oxygen are also well-known in the medical community as agents for treating blood and human tissue in order to fight disease or other pathogens. For example, ozone has been found, *e.g.*, to be effective

against various viruses and fungi, and to inactivate a wide variety of bacteria including *pseudomonas aeruginosa, staphylococcus aureus* and *mycobacterium tuberculosis*.

One very important use of reactive oxygen, *e.g.*, ozone or singlet oxygen, is in the sterilization of blood. Through the use of ozone and singlet oxygen, it is possible to deactivate a variety of bacterial and viral contaminants of blood, including HIV-1 and Hepatitis. Oxygenation in this manner may be used to treat blood prior to its transfusion. It is also known to oxygenate whole blood, by absorbing molecular oxygen into it, for various medical and surgical procedures such as coronary bypass operations.

The introduction of reactive oxygen gas molecules, such as ozone and singlet oxygen, to a biological fluid such as human blood, can be a problematic process. It is desirable to have the gas molecules introduced as efficiently as possible to the carrier liquid in order to minimize time, expense, and toxic effects. However, introduction of these gas molecules through the use of certain prior art technology can readily cause a formation of bubbles. This is particularly undesirable when blood is involved, since it can lead to hemolysis--the destruction of the cell walls of the red blood cells and the subsequent loss of hemoglobin therefrom. Thus a basic problem posed in the art, is how to properly and effectively introduce required quantities of reactive oxygen, *e.g.*, ozone or singlet oxygen, into a biological liquid in an efficient and safe manner, so that *e.g.*, in the important case of blood, reasonably high volume aliquots can be treated in a relatively short period of time without causing bubbling and consequently hemolysis.

In addition to the mechanical denaturation of cells or proteins that can accompany contact with gas, excessive contact with reactive oxygen may also degrade the biological material being purified. However, if the contact with reactive oxygen is insufficient, it will be ineffective to achieve any measure of sterilization.

Several techniques for effecting treatment of blood with ozone are described in U.S. Patent No. 4,632,980 by Zee. These include contacting the blood or blood product (*i.e*, serum or the like) with the ozone by passing the fluid medium as a film through a chamber containing the ozone, or by passing the blood through porous hollow fibers which are present in a chamber containing the ozone. The technique wherein the blood is contacted as a film is exemplified in this patent by the use of rotating vessels such as rotating bottles, which act to form the blood into a relatively thin film over most of the bottle interior surface, with the ozone being passed through the bottle interior. The concentration of ozone in the treating atmosphere is said to be in the range of about 1 to 100 ppm, and usually in the range of from about 1 to 20 ppm. In the actual experimental system described, the ozone level was at approximately 2 ppm, and apparently was even lower since the ozone was mixed with filtered air before being contacted with the blood in the rotating bottle. The patentee emphasizes the need for what are referred to as "mild" contact conditions, apparently referring to the very low concentrations of ozone which are used.

A difficulty encountered in the method and apparatus contemplated in the 4,632,980 patent is that the treated film of blood is upon continued rotation of the bottle remixed with the untreated blood, thereby continually nullifying in whole or in part any salutary effects which are otherwise achieved. It will be further appreciated that the film of blood formed can at best be contacted at one surface thereof, *i.e.*, the film resides on the bottle surface and is not amenable to contact with the ozone from the support side.

It has also been known in the prior art to utilize other methodology to oxygenate blood for several of the purposes previously discussed. In Jones, U.S. Patent No. 2,287,901, for example, blood is sprayed into a contained oxygen atmosphere so that the blood in the form of spray droplets may be exposed to the said atmosphere. This patent, incidentally, also illustrates oxygenation of a biological

liquid for oxygenating a patient's blood during a surgical procedure or the like, *i.e.*, oxygenation here is with O_2.

In commonly assigned U.S. Patent No. 5,366,696 by Williams, improvements in oxygenation apparatus are disclosed, for oxygenating a biological liquid by spraying. In such apparatus a chamber is provided for receiving and collecting the liquid (such as blood) which is to be contacted with the oxygenating gas, such as oxygen containing ozone. The oxygenating gas is introduced into a spray orifice in such a manner that the venturi effect generated by flow of the gas causes a flow of blood to be induced into and contacted with the gas stream, and to issue from the nozzle orifice as a dispersed spray of liquid droplets. A spray collection surface is located within the chamber in the spray path of the liquid droplets, which collects substantially the entirety of the droplets of oxygenated liquid. This collection surface is spaced from the spray orifice a distance selected so that the droplets impinge on the collection surface at relatively low velocity and form a film on the surface, where further contact with the gas can also be effected. The method of contact achieved in the said apparatus is very effective in that high energy mechanical impact of the droplets with the collection surface is avoided, whereby hemolysis effects are eliminated or greatly reduced. Much higher concentrations of ozone can be safely utilized in this apparatus than have heretofore been taught.

While apparatus of the type disclosed in the 5,366,696 patent has been found to be more effective than other devices and procedures for oxygenating biological liquids such as blood, it has nonetheless been found that in some instances there is still a low, but undesirable, degree of hemolysis because the blood or other biological liquid is still subjected to relatively high mechanical forces during the spraying process and during the impingement of the blood against the collection surface. A further and particularly important aspect of such oxygenation apparatus concerns the efficiency of gas transfer into the biological fluid.

The citation of any publication herein should not be deemed as an admission that such publication is available as prior art to the present invention.

SUMMARY OF THE INVENTION

The present invention advantageously provides a method for decontaminating a biological liquid while minimizing degradation of biological materials, such as proteins and cells. The method of the invention comprises contacting the biological liquid with sufficient reactive oxygen to deactivate microorganisms that may be present in the biological liquid by taking account of a reactive oxygen demand of the biological liquid. Critically, the maximum period of time, or effective treatment window, that the reactive oxygen is in contact with the biological liquid averts degradation of any biological material that may be present. The reactive oxygen demand of the biological liquid is a function of a reactive oxygen demand of a liquid carrier and a reactive oxygen demand of any biological material that may be present in the liquid carrier. The greater the reactive oxygen demand of the biological liquid, the greater the rate of reactive oxygen transfer needed to achieve the concentration to deactivate microorganisms within the effective treatment window. Thus, the period of time of exposure of a biological liquid to reactive oxygen will depend on the reactive oxygen demand of the biological liquid, the nature and amount of microorganisms present, and the rate of transfer of reactive oxygen from the gas into the biological liquid. The present invention advantageously provides for enhancing the rate of transfer of reactive oxygen from the gas into the liquid so that effective sterilization can occur with minimal degradation of biological material.

In particular, the invention provides for decontamination of viral pathogens. For example, the viral pathogen log titer can be decreased at least by a factor of 4; preferably, the viral pathogen log titer is decreased at least by a factor of 6. The present invention provides for treatment to destroy envelope viruses, surprisingly, non-envelope viruses, and, retroviruses. In specific embodiments, the viral

pathogen is selected from the group consisting of Porcine Parvovirus (PPV), Adenovirus, Infectious Bovine Rhinotracheitis (IBR) virus, and Bovine Viral Diarrhea (BVD) Virus, and Simian Immunodeficiency Virus (SIV).

According to the invention, in one embodiment the biological material is a protein, such as, but not limited to, a blood protein, for example immunoglobulin useful for passive immunization. The method of the invention is especially useful with a blood protein selected from the group consisting of serum albumin, clotting factor, and fibrinogen. The biological material may be a biological liquid containing proteins, such as but not limited to serum, plasma, cell culture fluid, liquid nutrient fluid, or liquid nutrient extract, *e.g.*, Bovine Pituitary Extract (BPE). In another embodiment, the protein may be isolated from a fermentation culture.

In a further aspect, the biological material comprises cells. For example, the invention provides for decontamination of any type of blood cell, such as but not limited to red blood cells, white blood cells, lymphocytes, or platelets. In a further embodiment, the cells are bone marrow cells, or stem cells. More particularly, the stem cells may be selected from the group consisting of peripheral stem cells, autologous marrow cells, allogeneic marrow cells, umbilical chord blood cells, and tissue culture cells. In one particularly useful embodiment of the invention, the stem cells are a $CD34^+$ or $CD38^+$ hematopoietic progenitor cells.

Thus, it is an object of the invention to provide a mechanism for determining the appropriate amount of activated oxygen for decontamination of a biological liquid.

Another object of the invention is to provide a mechanism for determining an activated oxygen contact window for effective decontamination without degradation of any biological material in a biological liquid.

Still a further object of the invention is to provide for rapid transfer of activated oxygen from a gas into a biological liquid.

These and other objects of the invention will be better understood by reference to the accompanying drawings and detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1. Perspective, simplified schematic view of an apparatus for use in the method of the present invention.

FIGURE 2. Phosphate buffered saline (PBS) ozone saturation curves at 22.5°C (open circles) and 21.5°C (open squares).

FIGURE 3. Ozone decay in saturated PBS at room temperature (22.5°C). The half-life was 273 sec (4 min, 33 sec); after 20 min, 3.1% of the ozone remained; the curve fits a third order polynomial equation with a coefficient (R) of 0.99992.

FIGURE 4. Effects of ozone concentration on ozone transfer into PBS and human IgG in PBS. Ozone concentration in PBS (y-axis, μg/ml) was determined by UV absorption at 260 nm after cumulative contact of 9 μg/ml ozone in air (ozone concentration) with PBS (solid circles); or 9 μg/ml ozone (solid squares), or 34 μg/ml ozone (solid triangles), with 0.5 μg/ml IgG in PBS.

FIGURE 5. Transfer efficiency of ozone in PBS and human IgG. Ozone transfer efficiency over time was determined from the data in Figure 4; the symbols are the same as in Figure 4. Ozone transfer efficiency was calculated as 100 x (amount of ozone in the liquid/total amount of ozone delivered in the gas).

FIGURE 6. Effects of ozone on 1% (w/v) human IgG in PBS. The IgG solution was contacted with 36 μg/ml ozone (gas concentration) for the period of time

indicated by a multiple pass technique. Ozone concentration in the solution was measured spectrophotometrically by absorption at 260 nm (A_{260}).

FIGURE 7. Effects of ozone on carbonyl formation in 1% human IgG/PBS solution in the presence of 1:5000 diluted octanol. Carbonyl concentration was determined by colorimetric reaction with 2,4-dinitrophenylhydrazine using standard techniques. The solution was contacted with 36 μg/ml ozone (gas concentration).

FIGURE 8. Effects of ozone on viral reduction of human IgG solutions in PBS, 10% maltose, pH 4.25, and 0.2 M glycine, pH 4.25. Three different viruses were analyzed by incubating the sample on an indicator cell line in serum free media, and scoring for cytopathic effect (CPE). The viruses tested were Adenovirus (vertical stripe bars), a non-enveloped virus; Infectious Bovine Rhinotracheitis (IBR) virus (horizontal stripe bars), an envelope virus; and Bovine Viral Diarrhea (BVD) virus (diagonal stripe bars), also an envelope virus.

FIGURE 9. Virus inactivation, as measured by viral titer, versus ozone concentration (μg/ml). (A) IBR inactivation versus ozone concentration. (B) BVD inactivation versus ozone concentration. (C) Adenovirus inactivation versus ozone concentration.

FIGURE 10. Virus inactivation, as measured by viral titer, versus increasing dilution in ozonated PBS. (A) Dilution of IBR with ozonated PBS. (B) Dilution of BVD. (C) Dilution of Adenovirus. (D) Dilution of Porcine Parvovirus (PPV).

FIGURE 11. Viral inactivation in 0.5 mg/ml IgG relative to pH in 0.2 M glycine containing octanol (1:5000). The ozone concentration was 34 μg/ml at a flow rate of 500 ml/min and a negative pressure of 2" of water. (A) Inactivation studies with Bovine Viral Diarrhea (BVD) virus. (B) Inactivation studies with Infectious Bovine Rhinotracheitis (IBR) virus. Viral inactivation, as determined by viral

titer, was evaluated over time of exposure. Control viral samples were prepared in PBS at pH 7.2 (solid circles). Samples containing IgG (0.5 mg/ml) were prepared in pH 7.2 (open circles) and pH 4.25 glycine-PBS (closed squares). Note that the suppression of viral inactivation due to IgG ozone demand (open circles) was completely eliminated, and viral killing enhanced, in the lower pH solution.

FIGURE 12. PBS saturation using pressurized ozone over time. Ozone (34 μg/ml) was contacted with PBS using a oxygenating device as shown in Figure 1 after multiple passes at ambient atmospheric pressure (data not shown), or with a back-pressure of +1 psi (open circles with a dashed line), +2 psi (open squares, solid line), or +3 psi (open triangles, dotted line). At higher pressure, ozone delivery rate was increased.

DETAILED DESCRIPTION OF THE INVENTION

As noted above, the present invention provides a method for sterilizing a biological liquid comprising contacting the biological liquid, preferably containing a surfactant and/or a cosurfactant, with a concentration and pressure of reactive oxygen for a time effective to deactivate contaminants present within the biological liquid. It has been discovered that the amount of reactive oxygen required to deactivate a microorganism in a biological liquid depends on the reactive oxygen quenching activity of the liquid carrier (*e.g.*, PBS, saline, or water), the quenching activity of any biological materials to be decontaminated or sterilized by reactive oxygen, and the type and amount of microorganism present. These quenching activities are referred to herein as reactive oxygen demand. Fully effective sterilization occurs when enough reactive oxygen is provided for a sufficient time to inactivate any microorganisms contaminants, while overcoming, or satisfying, the reactive oxygen demand of the biological liquid. The invention further relates to the discovery that minimizing the time to transfer reactive oxygen into a biological liquid containing a biological material and a microorganism reduces the

structural degradation of the biological material, particularly proteins, polypeptides, amino acids, and cells.

Examples of biological material that can be decontaminated according to the methods of the invention include protein preparations and cells. In one embodiment, the protein is serum immunoglobulin obtained from an immunized subject for use in passive immunotherapy. In another specific embodiment, the biological material comprises $CD34^+$ or $CD38^+$ hematopoietic stem cells.

As used herein, the term "sterilizing" refers to the deactivation of microorganisms present in a biological liquid.

The term "degradation" as used herein refers to an irreversible change in the physical-chemical or functional properties of a biological material. Examples of degradation include denaturation, fragmentation, or derivitization by reaction with oxygen. For example, a protein may become unfolded; it may be cleaved at a peptide or other bond into fragments; or amino acid side chains may be oxidized. Under such circumstances, the protein may lose its physiological activity. For example, an immunoglobulin may fail to bind antigen, or may not be recognized for opsonization via Fc receptors.

It should be appreciated that as used herein the term "biological liquids" (or "fluids") encompasses a wide variety of fluid substrates which may be oxygenated, and in some instances, sterilized by use of the invention. The biological liquid may, but need not necessarily, contain a biological material in solution or suspension. The term biological liquid includes, for example, blood and blood fractions, including whole blood, serum, plasma, packed red blood cells, platelets, and leukocytes; and as well, bone marrow and semen. Also included are fluids that may be administered to mammals, including humans, such as saline or glucose intravenous solutions and the like; and fluid nutrient media such as are employed to preserve organs for transplantation, or to grow bacteria, viruses, cells

(particularly cells for transplantation, fermentation to produce recombinant proteins, or hybridomas for production of monoclonal antibodies), parasites, and the like. Thus, the term further refers herein to any liquid containing a biological material, such as but not limited to proteins, carbohydrates, or cells suspended in a buffered solution, *e.g.*, immunoglobulin in phosphate buffered saline (PBS). The term also refers to such liquid carriers without any biological material; *i.e.*, the invention provides for sterilization or decontamination of a buffer solution or other liquid prior to admixture with a biological material. Finally, as used herein, the term "biological liquid" also refers to a liquid intended for *in vivo* administration, such as a fluorocarbon liquid for artificial blood or direct oxygen transfer in the lungs.

The term "biological material" is used herein to refer to proteins (including glycoproteins, proteoglycans, recombinant proteins, etc.), polypeptides, amino acids, carbohydrates, lipids (including lipid structures such as liposomes, micelles, lipoprotein particles, etc.), cells, and combinations of two or more of the preceding, which may be dissolved or resuspended in a biological liquid. The term biological material refers to the product to be sterilized, and thus excludes the microorganism being inactivated by oxygenation treatment.

The term "microorganism" as used herein refers to the contaminant to be inactivated by oxygenation treatment. Examples of microorganisms include the prion infectious element (which, though it may not be an "organism" in the traditional sense, clearly has pathogenic potential and can be transmitted through some mechanism), viruses (including envelope, and surprisingly, non-envelope viruses, and retroviruses), bacteria, fungi, and parasitic protozoa (such as plasmodium, trypanosomes, leishmania). Preferably, the microorganism is a known pathogen, such as HIV or Hepatitis. Other microorganisms may be opportunistic pathogens, such as certain bacteria or viruses that are normally inactivated by the immune system but are pathogenic in immune-compromised individuals. In some instances, the microorganism may not be pathogenic or

harmful, but is nevertheless an undesirable contaminant. An example of this last sort of microorganism is mycoplasma, which is a highly undesirable contaminant of cell cultures.

The term "oxygen" as used herein is meant to encompass all of these forms of the gas, and references to "oxygenation" similarly encompasses treatment by any of such forms of the gas and the transfer and absorption of the treating oxygen to the biological liquid, *e.g.*, for the sterilization of one or more components of the biological liquid.

The term "oxygenation treatment" or "oxygenating" as used herein refers to treatment of a biological liquid and/or microorganism with a reactive oxygen molecule. The reactive oxygen molecule can be supplied as a component of other gasses, such as normal (triplet) oxygen, nitrogen, air, etc. Preferably, and in the examples, *infra*, the reactive oxygen molecule supplied to the biological liquid is ozone (O_3). In another embodiment, the reactive oxygen molecule is singlet oxygen ($^1O_2$). Other examples of reactive oxygen molecules are well known in the art, and include molecules that can form oxygen radicals as well as activated oxygen, including biological or chemical substrates that can be treated to form reactive oxygen. For example, ozone can react to form reactive oxygen intermediates, *e.g.*, peroxides, which may then form singlet oxygen, react on their own, or form other reactive oxygen intermediates. In a specific aspect, the present invention contemplates supplementing the biological liquid with reagents that activate oxygen to a reactive state. For example, amino acids, particularly cysteine, efficiently convert ozone to singlet oxygen. Unsaturated carbon compounds, such as lipids and detergents, may enhance peroxide formation. This means that every protein has an intrinsic, composition dependent, singlet oxygen potential. The singlet oxygen potential for a given biological liquid can be increased by including free amino acids with high efficiency for ozone to singlet oxygen conversion.

The present invention is based, in part, on the following discoveries:

Every biological material has a characteristic "reactive oxygen demand" (somewhat akin to a $p[O_3]$); or anti-oxidant potential. In a biological liquid, reactive oxygen demand will be a function of the intrinsic reactive oxygen demand of the biological material plus the amount of reactive oxygen required for effective microorganism inactivation. In other words, the present inventors have discovered that effective sterilization requires that a sample or preparation is contacted with a sufficient concentration or quantity of reactive oxygen to satisfy that demand and supply enough reactive oxygen to destroy microorganisms. In essence, the invention applies rapid gas transfer kinetics to provide a pathotoxic dose of reactive oxygen in the presence of reactive oxygen quenchers (the liquid and a biological materials). Accordingly, the invention advantageously avoids saturating the biological liquid and biological material with reactive oxygen, which will occur as the exposure time increases, while at the same time killing microorganism. The amount of reactive oxygen will vary dramatically with the selection of different biological materials for sterilization, in addition to consideration of the reactive oxygen demand of the liquid carrier, and the kind and amount of microorganism to be destroyed. As described below, various means are available to effectively provide a sufficient reactive oxygen concentration for sterilization without denaturing or destroying the biological material being sterilized.

The inventors have also discovered that damage or deactivation of the biological material being sterilized is predominantly a function of the time of exposure to reactive oxygen. This value is specific for the material being sterilized. Most importantly, it has been found that even sub-effective doses of reactive oxygen over long periods of time can denature proteins or damage cells. Of course, treatment over long periods with a microorganism-deactivating dose of reactive oxygen can also damage the biological material. Thus, in part, the present invention concerns enhancements to the efficiency of reactive oxygen transfer to a biological liquid to satisfy the reactive oxygen demand in the shortest period of

time, thus effecting the inactivation of microorganisms without significantly destroying the biological material. This exposure time is the "effective treatment window." Shorter times fail to deactivate microorganisms. Longer times lead to unacceptable degradation.

Further discoveries relate to the effects of temperature, pH, surfactants and cosurfactants, and pressure on the efficiency of reactive oxygen transfer.

The inventors herein have found that any biological material preparation, whether purified proteins (such as immunoglobulins or albumins), isolated proteins (such as plasma or serum), carbohydrates, lipids, cells, or combinations thereof, can be sterilized by determining the reactive oxygen demand for the preparation, and determining the permissible reactive oxygen contact time range and concentration range to achieve sterilization without degradation of the biological materials. Where necessary, *e.g.*, to achieve a high saturation point of reactive oxygen in a biological liquid with a high reactive oxygen demand, the present invention provides for adjusting variables such as temperature differential between the gas and the biological liquid; pH of the biological liquid; inclusion of a surfactant and/or cosurfactant in the biological liquid; gas pressure; and gas humidity to increase transfer efficiency.

Thus, in one aspect the invention is directed to a method for sterilizing a protein preparation comprising contacting the protein preparation with a concentration of reactive oxygen for a period of time, which concentration and time period depend on the concentration of protein and the reactive oxygen demand of the protein and liquid carrier that makes up the preparation, such that a total amount of reactive oxygen with which the protein preparation is contacted is sufficient to destroy a microorganism (prion or prion-like proteins, virus, bacteria, fungus, protozoan parasite) but does not degrade all or most of the protein, and leaves the protein physiologically active.

Apparatus for Practicing the Invention

The principles of the present invention can be applied to any current technology for introducing ozone into a biological liquid. Modifications to such technologies are necessary to render them capable of meeting the criteria set forth herein. For example, operation parameters of a device as described in Williams, U.S. Patent No. 5,366,656 can be modified according to the invention. In a preferred aspect, ozone can be generated as described in U.S. Patent No. 5,094,882, issued March 10, 1992 and U.S. Patent No. 5,443,800, issued August 22, 1995.

In a preferred embodiment, an apparatus for effectively oxygenating a biological liquid provides an open mesh surface having opposed accessible faces. Flow means are disposed to establish a flow of the biological liquid along the mesh surface, thereby forming the fluid into a membrane at the mesh openings of the surface. Means are additionally provided for establishing an oxygenating gas atmosphere in contact with the opposed faces of the mesh surface, for oxygenating the biological liquid membrane from both sides of the mesh surface; and means are provided for collecting the flow of oxygenated biological liquid from the mesh.

The preferred apparatus provides for maximum efficiency of contact of the biological material with oxygen or reactive oxygen by exposing both surfaces of a liquid membrane to the sterilization gas. Furthermore, in a preferred embodiment, the apparatus places minimal mechanical stress on the material, since the liquid moves by controlled gravity flow down the mesh. Spraying and other harsher techniques are thus avoided. More importantly, the time of exposure of the material to reactive oxygen is minimized because the apparatus provides for highly efficient reactive oxygen transfer.

The mesh surface may be formed as a cylinder, which can be mounted with its axis vertically directed. The means for flowing the biological liquid can in this instance comprise a distribution cap secured at the top of the cylinder with its periphery engaging the end of the cylinder. A flow of biological liquid is

provided onto the cap to enable a gravitational flow toward the edge of the cap and thereby onto the mesh surface. The cylinder and flow means are mounted within a reaction and fluid collection chamber. The chamber is provided with a gas inlet for oxygenating gas and a spaced gas outlet for discharging the gas. Also connected to the chamber are fluid inlet means for the biological liquid to be treated, and an outlet enabling the treated biological liquid to be collected.

The flow means may further comprise a fluid delivery conduit and a flow control means between the conduit and distribution cap for regulating the amount of flow to the mesh surface. The mesh surface preferably comprises a material which is inert to the biological liquid and to the oxygenating gas used in the apparatus, and may be in the form of a screen, a webbing or the like.

The preferred apparatus of the invention, which in a preferred embodiment is described in co-owned, copending application Serial No. _____, Attorney Docket No. 1138/1/021, filed on even date herewith, and entitled "APPARATUS AND METHOD FOR OXYGENATING BIOLOGICAL LIQUIDS", which is incorporated herein by reference in its entirety. Figure 1 shows an embodiment of the invention.

In Figure 1, an example of the apparatus 10 comprises an outer substantially hollow cylindrical chamber 12. The bottom of chamber 12 is provided with a liquid exit port 13 for withdrawing biological liquids following the oxygenation. Mounted coaxially within chamber 12 is a an open mesh surface 14 which is formed as a hollow open-ended cylinder 15. The cylinder may be supported in the manner shown by being secured to and suspended from a flow distribution cap 21. The latter is in turn mechanically supported by the gas input conduit 19. Mesh 14 is an open webbing or screen relatively resistant to the oxygenating gas being used and to the biological liquid, such as gauze comprised of natural or synthetic materials; plastics such as polyethylenes, PVCs and the like; as well as highly inert metals and alloys. The mesh generally has uniform circular, rectangular or

other openings, with a mean opening dimension in the range of from about .02 to 10 mm. The chamber 12 is generally enclosed at its upper end by a cover 16 through which passes (in sealed relation) a liquid inlet port 17 and a gas exit port 18. Input port 17 in turn supports in sealed relation the coaxially mounted gas input conduit 19 for oxygenating gas which is to be introduced into the apparatus. Conduit 19 is joined to a gas dispersing nozzle 20 which disperses the oxygenating gas to the upper interior of cylinder 15. The oxygenating gas is provided to the conduit 19 from a conventional source as, for example, the apparatus described in commonly assigned Dunder, U.S. Patent No. 5,094,822. It passes into chamber 12 at the interior of cylinder 15, and flows as shown by arrows 22 in an axial direction toward the bottom of cylinder 15, thence passes upwardly between the cylinder 15 and the interior wall of chamber 12, and exits from port 18.

The flowable biological liquid to be treated, which may representatively be considered as whole blood, is provided to inlet port 17 and flows to and over flow distribution cap 21, the periphery of which is smoothly adjoined by mechanical, adhesive or other means to the upper edge 24 of the mesh 14 comprising cylinder 15. A liquid flow control or regulator 25 is positioned between inlet 17 and distributor cap 21. By moving body 26 axially toward or away from cap 21 a valve action is achieved which increases or decreases the flow of liquid in accordance with the requirements of a particular application. Body 26 may be threadingly mounted on inlet 17 to enable this action or may be vertically displaced by simple electromechanical means such as solenoid actuation or the like, which opens into the interior of chamber 12. As the liquid flows it is formed into a very thin membrane -- typically this membrane can be of the order of 50 to 750 microns thickness -- in contact with the gas on both surfaces. In consequence a very effective degree of gas mass transfer is achieved.

Anti-oxidant Potential (Reactive Oxygen Demand)

The present invention is directed in an important aspect to identification of the concept of reactive oxygen demand. Reactive oxygen demand is, as described

above, a concentration of reactive oxygen adsorbed or neutralized by a biological material, such as a protein, protein preparation, or a cell preparation. The value for reactive oxygen demand varies with the makeup of each biological material. Different proteins or protein preparations, for example, have unique values for reactive oxygen demand.

As shown in the examples, *infra*, reactive oxygen demand of a biological material can be empirically determined by titrating the amount of reactive oxygen required to reach saturation of a solution or suspension. In any system, this will depend on time of exposure and the concentration and pressure of reactive oxygen, as well as temperature, pH, and the presence of surfactant and/or cosurfactants. As discussed below, where the reactive oxygen demand of a biological liquid requires exposure times that exceed safe limits to avoid degradation of the biological material, various means to increase transfer efficiency can be employed.

Means for Increasing Reactive Oxygen Transfer Efficiency

As discussed above, the present invention provides a number of different means for increasing reactive transfer efficiency, including adjusting the pH of the biological liquid; providing a temperature differential between the gas and the liquid; increasing the pressure of the gas and the concentration of reactive oxygen in the gas, or both; and including a surfactant and/or cosurfactant in the biological liquid. These strategies may be employed individually, *i.e.*, in isolation, or two or more can be employed simultaneously. It should be noted that merely adding a biological material to the biological liquid facilitates reactive oxygen transfer.

Although the invention is not limited to any particular theory of operation, in the absence of a solute or material suspension in the liquid, or some other tension on the system, reactive oxygen concentration builds up on the surface of the biological liquid, creating a reactive oxygen barrier to further gas transfer. The presence of a biological material disrupts formation of such a barrier. The factors discussed below also help overcome formation of a reactive oxygen barrier.

Adjusting pH. As shown in the examples, *infra*, as pH is decreased from physiological pH (about pH 7.2) to close to pH 4, the rate of ozone transfer increases dramatically. Hence, in a preferred aspect, pH of the biological liquid is maintained between pH 4 and 7. In a preferred aspect, pH is closer to pH 4, *e.g.*, pH 4-5. In a specific embodiment, *infra*, the pH is 4.25.

Temperature differential. It has been found that maintaining a temperature differential of up to 5°C between the gas phase (at the higher temperature) and the biological liquid increases reactive oxygen transfer efficiency. Preferably, the absolute temperature of the system is regulated between about 4°C and about 40°C; it may be up to 42°C. In specific embodiments, the gas and liquid temperatures are within about 5°C of room temperature (22.5°C); in another embodiment, gas and liquid temperatures are within about 5°C of 37°C.

Pressure. Molecular mass transfer considerations promote increased reactive oxygen transfer as the concentration (*i.e.*, partial pressure) of reactive oxygen in the gas is increased. It has also been found that increasing the gas pressure by up to 5 psi, and preferably up to 3 psi, above ambient or atmospheric pressure increases the efficiency of reactive oxygen transfer into a biological liquid.

Surfactants and cosurfactants. In a preferred aspect, the protein preparation includes a surfactant and/or cosurfactant. The presence of a surfactant and/or cosurfactant greatly facilitates transfer of reactive oxygen into the substrate, *i.e.*, the protein preparation. It has been found that the transfer rate efficiency reaches a plateau after the first one to three passes in a multiple run system, then generally decreases. By including a surfactant and/or cosurfactant, the transfer rate efficiency remains steady at the plateau level; there is no apparent decrease.

In a presently preferred embodiment, the cosurfactant is octanol. Octanol is closely related to octanoic acid, a blood resident fatty acid. The octanol may be oxidized to octanoic acid by the reactive oxygen. Other surfactants, particularly

C-6 to C-18 alcohols or acids, or lecithins, can also be used. In a further specific embodiment, the surfactant is sodium dodecyl sulfate (SDS). Any surfactant that is inert to reactive oxygen or that does not adversely affect the biological material can be employed. In a particular aspect, preferably, the surfactant chosen corresponds to, or may react with reactive oxygen to form, a naturally occurring compound.

Although not intending to be bound by any particular theory, it is believed that the presence of a surfactant and/or cosurfactant prevents protein (or other molecular) clumping, thus maintaining protein accessibility and avoiding excessive reactive oxygen demand effect. The surfactant also decreases surface tension of the biological liquid, permitting faster gas transfer into the liquid.

Surfactants are especially useful in film-type reactive oxygen contactor apparatuses, *e.g.*, as described above and shown in Figure 1. The presence of a surfactant advantageously enhances liquid flow characteristics.

Treatment of Proteins

The various kinds of protein that can be treated according to the invention include proteins produced in fermentation of recombinant or non-recombinant microorganisms; animal proteins; and preferably, human proteins -- more preferably, blood proteins and blood products. Examples of suitable proteins include immunoglobulin (as shown in specific examples, *infra*), serum albumin, insulin, clotting factors (Factor VIII, Factor IX, Factor X), fibrinogen, prothrombin and thrombin, interferons, lymphokines, cytokines, and the like.

According to the invention, a protein preparation is treated with reactive oxygen in an appropriate apparatus for a time and at a concentration to saturate the preparation without denaturing the protein. Effective reactive oxygen transfer occurs when the amount of reactive oxygen is sufficient to deactivate or destroy microorganisms, such as viruses, but does not destroy protein function. For

example, effective reactive oxygen transfer for serum IgG destroys viral pathogens present in the preparation, while retaining IgG antigen-binding activity. Preferably, this is accomplished in a single run of less than three minutes exposure to ozone, and more preferably less than two minutes exposure.

As can be appreciated by one of skill in the art, the optimum range for reactive oxygen treatment is different for each type of protein or protein preparation. It can be readily determined by contacting the protein with reactive oxygen to the saturation point for various time periods, and testing for protein activity. Once the reactive oxygen demand of the protein or protein preparation is determined, the invention provides for effecting reactive oxygen transfer within the required time period to avoid protein denaturation, as described in detail, *infra*.

Protein structural integrity or activity can be analyzed by a number of criteria, including but not limited to: polyacrylamide gel electrophoresis, isoelectric focusing, amino acid content, functional activity (such as enzymatic activity for an enzyme, antigen binding or complement activation for an immunoglobulin), antigenicity (*i.e.*, recognition by an antibody), HPLC analysis, sequence analysis, differential scanning calorimetry, and other techniques for evaluating protein activity.

Specific decontamination conditions can be determined for proteins by considering the concentration of protein in the preparation, the inherent reactive oxygen demand of the protein, the actual reactive oxygen demand (inherent demand times concentration), and the reactive oxygen requirement for decontamination of microorganisms, *e.g.*, viruses. Examples of decontamination conditions and analytical criteria for confirming retention of protein function for various proteins preparations are provided in the specific examples, *infra*.

Decontamination of Cells

Various cell preparations are suitable for decontamination according to the claimed invention, including but not limited to whole blood, packed red blood cells (RBCs), white blood cells, lymphocytes, platelets, bone marrow, and stem cells. In a preferred aspect, the present invention provides for decontamination of stem cells, such as peripheral blood or whole blood stem cells, autologous stem cells, allogeneic stem cells, and chord blood stem cells.

Thus, in another embodiment, the invention provides for decontamination of a cell preparation comprising contacting the cells with a concentration of reactive oxygen for a period of time, which concentration and time period depend on the density of cells and the reactive oxygen demand of the cells, such that the total amount of reactive oxygen with which the cell preparation is contacted is sufficient to destroy a viral pathogen or other contaminant but does not denature or destroy the cell. In this embodiment, the apparatus shown in Figure 1 is preferred. An apparatus of the invention for the decontamination of cells must avoid mechanical damage to the cells. The apparatus of Figure 1 is particularly suited for this.

In connection with decontamination of cells (or of certain fluids like plasma and serum), various anti-oxidant enzymes, such as glutathione in red blood cells, and catalase in serum, may be present that increase the anti-oxidant potential, and hence the reactive oxygen demand, of such preparations. However, the present invention advantageously provides for neutralizing the anti-oxidant effects of these enzymes to achieve inactivation of microorganisms without destruction of the cells.

The role of reactive oxygen demand in reactive oxygen-mediated decontamination has important implications for sterilization of cells as well. Thus, the present invention provides for determining the reactive oxygen pressure, exposure time, cell concentration, and other factors, accounting for any inherent cellular reactive oxygen demand, to decontaminate cells. As discussed below, once the reactive oxygen demand for saturation of a particular cell preparation is determined,

various means can be employed to ensure adequate reactive oxygen transfer efficiency to meet that demand without incurring cellular degradation.

Particularly preferred for treatment according to the invention are autologous or allogeneic $CD34^+$ or $CD38^+$ hematopoietic stem cells. Such cells may be obtained and stored prior to radiation or chemotherapy, which destroys these types of cells in a patient. The harvested $CD34^+$ or $CD38^+$ cells are then treated to decontaminate any viral pathogens or other microorganisms before transplantation into the immune-compromised patient.

Decontamination of cells involves treatment of complex structures with many variables. Accordingly, as discussed herein, in preferred aspects of the invention, various strategies may be employed to enhance reactive oxygen treatment of cells.

Antioxidant. In one embodiment, a biological liquid containing cells is supplemented with an effective concentration of amount of an antioxidant. The presence of an antioxidant will, of course, affect the reactive oxygen demand of the biological liquid. Its presence can serve to protect cells to a greater degree than any microorganisms that might be present. As shown in a specific example, *infra*, the presence of an antioxidant protects platelets from degradation during ozone treatment.

Examples of antioxidants for use in the present invention include, but are not limited to, vitamin-C, vitamin-E (preferably a water soluble form of vitamin-E), glutathione, and D-mannitol. In a specific embodiment, the antioxidant is D-mannitol. The concentration of antioxidant to be used will depend on the concentration and nature of the cells in the biological liquid, but will generally be within accepted effective concentration ranges, which are well known for these antioxidants. Alternatively, an effective concentration can be determined by simple titration analysis.

Humidity. High humidity of the gas is important for the decontamination or sterilization of a cell preparation (it may also be of value for a highly concentrated protein or lipid preparation), to prevent drying of the cells or denaturation of the proteins. Accordingly, in a preferred aspect decontamination of a biological liquid containing cells in accomplished by contacting the biological liquid with a gas having 100% humidity. Increased humidity has also been found to be necessary to compensate for increasing the ozone concentration, which has a drying effect on the biological material. Increased humidity has little effect, however, on liquids containing up to 20% of most proteins.

Other Biological Materials

As can be readily appreciated, the principles set forth above for decontamination and sterilization of proteins, protein preparations (including fluids such as plasma, serum, semen, etc.), and cells apply as well to preparations containing carbohydrates, lipids (including micelles and liposomal vesicles), and similar materials.

Of particular interest in this regard are total nutrient admixtures for total parenteral nutrition, *e.g.*, as described in Deitel et al. [*CJS* 32:240-243 (1989)]. These mixtures contain lipid particles, liposomes, proteins (*e.g.*, heparin), amino acids, carbohydrates, vitamins, and minerals. Using the present invention, the reactive oxygen demand of such a total nutrient admixture can be determined. The admixture can be treated to deactivate any microorganisms under conditions that protect the components. If necessary, pH can be easily adjusted after treatment with appropriate buffers or salts.

Viruses

Various types of viruses are contemplated for deactivation or destruction using the methods of the present invention, including envelope (lipid) and, surprisingly, non-envelope (non-lipid) viruses. In addition, the invention advantageously provides for deactivation of retroviruses. Examples of viruses, and the log viral titers of

deactivation achieved with the present invention, include the non-envelope viruses Procine Parvovirus (PPV) (5.00) and Adenovirus (8.00); the envelope viruses Infectious Bovine Rhinotracheitis (IBR) virus (8.10), and Bovine Viral Diarrhea (BVD) virus (7.00); and the retrovirus Simian Immunodeficiency Virus (SIV) (6.00).

Of particular interest for the present invention is the deactivation of pathogenic viruses that contaminate the blood supply, or that are pathogenic in humans. Accordingly, the present invention provides for deactivation of Human Immunodeficiency Virus (HIV), including HIV-1 and HIV-2; Human T-Lymphotrophic Virus (HTLV), including HTLV-1 and HTLV-2; Hepatitis Virus, including Hepatitis-B, Hepatitis-C, and all Hepatitis strains; Adenovirus; Herpes Virus, including all strains; Cytomegalovirus; Epstein-Barr Virus; etc.

The present invention may be better understood by reference to the following non-limiting Examples, which are provided as exemplary of the invention.

EXAMPLE 1: Determination of Ozone Demand of Solutions

Materials and Methods

Apparatus. Ozone was generated in an apparatus as described in U.S. Patents No. 5,094,822 and No. 5,443,800. This patented system is microprocessor controlled, and provides significant control over ozone concentration in the gas. All ozone gas concentrations reported in the following examples were calculated from the settings on the apparatus. Unless otherwise stated, materials were contacted with 34 μg/ml of ozone (gas concentration) in oxygen.

The biological liquid was contacted with ozone in an apparatus as shown in Figure 1. The liquid flow rate in the device was 25 ml/minute. Polyvinyl chloride (PVC) mesh having roughly square pores of an average size of 3 mm was used to form liquid membranes. Total contact time with ozone was 1 minute. Longer

times were achieved using multiple runs. In some cases, the apparatus was fitted with a back-pressure device to increase the gas pressure by up to 3 psi.

Determination of ozone demand. For a basic determination of ozone demand, PBS or PBS containing a material (*e.g.*, a protein, such as IgG) is contacted with ozone. Extended ozone contact was achieved by passing the biological liquid through the device multiple times. Ozone concentration in the PBS was determined by absorbance at 260 nm (A_{260}). The ozone concentration saturation reflects the ozone demand.

Results and Discussion

As shown in Figure 2, saturation of PBS occurs after about 5 minutes. The level of ozone present for saturation is temperature dependent, as would be expected. Higher saturation occurs at a lower liquid temperature.

Figure 3 shows the ozone decay curve in PBS at room temperature. This information is useful to evaluate the ozone contact time for achieving an effective concentration in a biological liquid within the treatment window. Rapid ozone decay would mandate a longer exposure time to achieve effective ozone contact. Temperature greatly affects ozone decay rate, and must be considered when calculating contact times.

Including a protein greatly increases the ozone demand of a biological liquid, as shown in Figure 4. Here, the concentration of ozone required to achieve saturation increases greatly in the samples containing 0.5 mg/ml IgG. These data also show that the solution treated with a greater concentration of ozone (34 μg/ml gas concentration) achieved saturation more quickly. However, the transfer efficiency (calculated as 100 x amount of ozone in liquid/amount of ozone contacted to liquid) of ozone was greater at the lower ozone concentration (9 μg/ml gas concentration) as shown in Figure 5.

EXAMPLE 2: Effects of Ozone on Human IgG

Immunoglobulins in the plasma are important humoral factors to maintain immunity. The infusion of immune globulins into humans has led to immunologic improvement [Christensen *et al.*, *J. Pediatr.*, **118**:606-14 (1991)]. However, for patient infusion, intravenous IgG has to be sterile. IgG can be successfully sterilized with ozone under controlled conditions. To minimize side effects, the actions of free radicals produced by oxidative stress and ozonolysis on IgG suspended in biological fluids have to be determined [Pryor and Uppu, *J. Biol. Chem.*, **268**:3120-6 (1993)]. In this example, the ozone effects of protein degradation in human IgG were examined by polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting, and relative changes in amino acid composition of IgG. Carbonyl groups resulting from various oxidative conditions were also determined.

Materials and Methods

Ozone treatment was performed as described in Example 1. Immunoglobulin was prepared at 1% in PBS with 1:5000 octanol. Polyacrylamide gel electrophoresis was performed in gradient gels (4-20% acrylamide). Carbonyl content was measured by the hydrazine reaction technique [*Methods Enzymol.*, **186**:464 (1990)]. Complement activation was determined by a standard assay method [*Manual of Clinical Laboratory Immunology, Third Edition*, N.R. Rose et al., American Society for Microbiology (1986), pp. 175-184]. Immunoblotting experiments were performed by electroblotting viral antigen proteins onto nitrocellulose. The ozone treated sample was incubated with the nitrocellulose. The nitrocellulose was washed, and then incubated with an anti-human-Ig conjugated with alkaline phosphatase. The nitrocellulose was washed again and developed with alkaline phosphatase substrate solution (5-bromo-chloro-3-indoyl phosphate and nitroblue tetrazolium) to reveal bound antibodies.

Results

Conditions for optimal ozone transfer efficiency were determined within an operating window of approximately two minutes. Figure 6 shows that ozone saturation of a solution of 1% IgG in PBS is achieved after about three minutes. Human IgG retained both structural and functional integrity, as demonstrated by SDS-PAGE, immunoblotting (with nitrocellulose-associated antigen), and amino acid analysis. There was no complement activation by IgG in this treatment window, indicating retention of Ig structural integrity.

Following treatment times in excess of two minutes, small quantities of protein carbonyl were detectable (Figure 7). Longer ozone treatment caused human IgG to aggregate in a dose dependant manner, probably as a result of protein cross-linking. As aggregation proceeded, the immunogenic reactivity decreased as detected by Western blotting. In addition, complement activation increased as a result of Ig aggregation and breakdown. A progressive loss of histidine and tyrosine residue concentration was measured by amino acid analysis of the IgG. Production of protein carbonyl groups increased steadily with ozone exposure (Figure 7), indicative of oxidative damage.

Conclusion

With careful use of controlled ozone applied within the correct operating window for ozone exposure, it is possible to sterilize human IgG. Contaminating microorganisms, such as bacteria and viruses can be deactivated without deleterious effect on IgG.

EXAMPLE 3: Viral Inactivation in Human IgG Solution by Oxidative Stress

Immunoglobulins confer humoral immunity in man. They have been widely used for infusions of IVIg (intravenous immunoglobulin) in therapy and prophylaxis as well as in patients with autoimmune diseases and immune deficiencies. To guarantee high purity, efficacy and safety of IVIg requires stringent viral and

bacterial inactivation which is becoming a global concern. A microprocessor controlled system, as described in Example 2, was used to deliver the exact, required concentrations of ozone into biological liquids. Treatment with ozone inactivates infectious agents present in IgG under conditions that retain the intrinsic protein biological activities. Using an operating window of from one to three minutes, IgG remained functionally and structurally intact as demonstrated by SDS-PAGE, immunoblotting and amino acid analysis with no evidence of IgG aggregation.

Two different buffers, 10% maltose and 0.2 M glycine, pH 4.25 to dissolve the human IgG were investigated for inactivation of 2 enveloped viruses, IBR and BVD and 1 non-enveloped virus, Adenovirus. These viruses were inactivated at concentrations of 10^7, 10^6, and 10^6 log reductions for IBR, BVD and Adenovirus respectively using the glycine, pH 4.25 buffer (Figure 8). The maltose buffer was less effective in viral inactivation probably as a result of the higher ozone demand by the disaccharide, and thereby reducing the sterilizing power of ozone (Figure 8).

EXAMPLE 4: Inactivation of Enveloped and Non-enveloped Viruses by Oxidative Stress

Ozone, an energized form of oxygen, has powerful oxidizing properties that have led to its use in deactivating viruses and bacteria in drinking water or effluent systems. While ozone has been considered for treating a variety of biological fluids, there has been a general perception that the technology is limited to enveloped viruses. For this reason, a non-enveloped and several enveloped viruses were selected to study ozone-derived virus inactivation using a simple ozone infusion model. PBS containing increasing concentrations of ozone was introduced to viral samples, and treated samples then assessed for virus titre. For the enveloped Infectious Bovine Rhinotracheitis (IBR) virus and Bovine Viral Diarrhea (BVD) virus dissolved ozone was necessary for complete inactivation of approximately 6 logs of virus (Figure 9A, B). Bovine Adenovirus type 7, a non-enveloped model, was totally inactivated at 2.2 μg/ml of dissolved ozone (Figure

9C), notably lower than the concentration required for deactivation of the enveloped viruses. Dilution of virus into ozonated PBS also showed inactivation of enveloped viruses IBR (Figure 10A) and BVD (Figure 10B), and non-enveloped viruses Adenovirus (Figure 10C), and PPV (Figure 10D). Results suggest that ozone is an effective agent for inactivating both enveloped and non-enveloped viruses, and retroviruses.

EXAMPLE 5: *In Vitro* Protection of Platelets From Free Radicals Produced After Ozone (O_3) Treatment

Free radicals produced from O_3 for viral deactivation of blood components, can be detrimental to platelets. Addition of D-mannitol to platelet concentrates was investigated as a protective agent.

Method

Whole blood was collected into a collecting device with citrate-phosphate-dextrose-adenine-1 (CPDA-1) and the platelet concentrate was resuspended in PBS with and without 100 mM D-mannitol. The platelet concentrations were treated with 10 μg/ml ozone. Total platelet count and mean platelet volume were determined by automated analyzer. Both samples were treated with oxygen (O_2) as a control.

Results

A 15% reduction in mean platelet volume was observed for platelet concentrates treated with O_3 in the absence of mannitol. Samples treated with O_2 and O_3 in the presence of mannitol showed no decrease. At 14 minutes, a 41% reduction was seen in O_3 processed samples with and without mannitol respectively while no decrease was noted in the O_2 controls. Total platelet counts fell 43% with O_3 treatment alone, 22% with mannitol added and 11% and 12% with O_2 controls.

Discussion

Mannitol protected platelet structure and number from free radicals produced as a result of ozone treatment. These data show the importance of controlling oxygenation to avoid cellular toxicity.

EXAMPLE 6: Effect of *In Vivo* Oxidative Stress on Human Endothelial Cells

Endothelial cells compose the lining of arteries and veins and are subject to a variety of insult from toxins circulating in the blood. This study was designed to study the *in vitro* effects of ozonated PBS on human umbilical vein endothelial cells (HUVEC) grown in culture. PBS was ozonated at 4°C, using two temperature conditions monitored continuously by digital thermometers. Endothelial cell monolayer 1 was kept on ice with a starting temperature of 4°C warming up to 8°C. Endothelial cell monolayer 2 was monitored at 10°C to 19°C. Control monolayers were exposed to 4°C PBS with no ozone. Cells were observed for cellular injury including rounding up, condensed nuclei and granular cytoplasm. Damage to the cells occurred in monolayer 1 at 8°C within 6 minutes, and in monolayer 2 at 16°C within 3 minutes. The control monolayers showed no changes. These results indicate that the viability of HUVEC cells can be maintained when the monolayer is exposed to ozonated PBS between 4°C to 8°C, within 10 minutes.

EXAMPLE 7: Effects of Ozone On The Hematopoietic Activity of Human Pluripotent Progenitor Cells

Pluripotent progenitor cells (stem cells) are capable of differentiating into a variety of cell lineages. Stem cell viability and differentiation is therefore of great importance in treatment of whole blood with ozone. Using the device in Figure 1 designed to deliver high concentrations of ozone to blood and other biological fluids in a controlled manner, the effect of ozone treatment on normal hematopoiesis (colony formation, proliferation and multi-lineage cell

differentiation) was examined using standard progenitor colony methylcellulose protocols. An enriched cell population was prepared using $CD34^+$ magnetic cell sorting (MACS). Colonies were scored and the plating efficiency calculated as compared to peripheral blood mononuclear cells (PBMC's) controls. Colony progenitors were also analyzed for CD34 and CD38 surface markers as well as cell viability using two color flow cytometry. Preliminary results have demonstrated extremely low levels of cellular damage at concentrations of ozone that achieve virtually 100% viral and bacterial deactivation.

EXAMPLE 8: Various Factors Affect Ozone Transfer Efficiency

The effect of pressure on ozone transfer rate was determined using an apparatus and methods as described in Example 1. As shown in Figure 12, increasing the total gas pressure significantly increased the rate of transfer of ozone into PBS, and the total amount of ozone in the ozone saturated PBS.

The present invention is not to be limited in scope by the specific embodiments describe herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and the accompanying figures. Such modifications are intended to fall within the scope of the appended claims.

It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

WHAT IS CLAIMED IS:

1. A method for decontaminating a biological liquid comprising contacting the biological liquid with an amount of reactive oxygen in a gas effective to deactivate microorganisms that may be present in the biological liquid, which amount of reactive oxygen satisfies a reactive oxygen demand of the biological liquid, wherein the reactive oxygen demand of the biological liquid is a function of a reactive oxygen demand of a liquid carrier and a reactive oxygen demand of a biological material that may be present in the liquid carrier; for a period of time that averts degradation of the biological material that may be present in the biological liquid.

2. The method according to claim 1, wherein the microorganism is a viral pathogen.

3. The method according to claim 2, wherein the viral pathogen log titer decreases at least by a factor of 4.

4. The method according to claim 3, wherein the viral pathogen log titer decreases at least by a factor of 6.

5. The method according to claim 2, wherein the viral pathogen is selected from the group consisting of an envelope virus, a non-envelope virus, and a retrovirus.

6. The method according to claim 2, wherein the viral pathogen is selected from the group consisting of Procine Parvovirus (PPV), Adenovirus, Infectious Bovine Rhinotracheitis (IBR) virus, Bovine Viral Diarrhea (BVD) virus, and Simian Immunodeficiency Virus (SIV).

7. The method according to claim 1, wherein the microorganism is selected from the group consisting of prion proteins, bacteria, fungi, and protozoa.

8. The method according to claim 1, wherein the biological material is a protein.

9. The method according to claim 8, wherein the protein is a blood protein.

10. The method according to claim 9, wherein the protein is immunoglobulin.

11. The method according to claim 9, wherein the protein is selected from the group consisting of serum albumin, clotting factor, and fibrinogen.

12. The method according to claim 1, wherein the biological material is a biological liquid containing proteins.

13. The method according to claim 12, wherein the biological liquid is selected from the group consisting of serum, plasma, and cell culture fluid.

14. The method according to claim 13, wherein the protein is isolated from a fermentation culture.

15. The method according to claim 1, wherein the biological material is a cell.

16. The method according to claim 15, wherein the biological liquid further comprises an antioxidant.

17. The method according to claim 16, wherein the antioxidant is D-mannitol.

18. The method according to claim 15, wherein the cell is a blood cell.

19. The method according to claim 17, wherein the blood cell is selected from the group consisting of a red blood cell, a white blood cell, a lymphocyte, and a platelet.

20. The method according to claim 15, wherein the cell is a bone marrow cell.

21. The method according to claim 15, wherein the cell is a stem cell.

22. The method according to claim 20, wherein the stem cell is selected from the group consisting of a peripheral blood stem cell, an autologous marrow cell, an allogeneic marrow cell, an umbilical chord blood cell, and a tissue culture cell.

23. The method according to claim 20, wherein the stem cell is a $CD34^+$ or $CD38^+$ hematopoietic progenitor cell.

24. The method according to claim 15, wherein the cell is an endothelial cell.

25. The method according to claim 15, wherein the gas has a humidity of 100%.

26. The method according to claim 1, wherein the biological liquid comprises a surfactant or co-surfactant.

27. The method according to claim 26, wherein the co-surfactant is octanol.

28. The method according to claim 1, wherein the biological liquid is at a lower temperature than the gas.

29. The method according to claim 1, wherein the biological liquid has a pH of between about 4.0 and 7.0.

30. The method according to claim 29, wherein the pH of the biological liquid is between about 4.0 and 5.0.

31. The method according to claim 30, wherein the pH of the biological liquid is 4.25.

32. The method according to claim 1, wherein the pressure of the gas is from 1 to 5 psi above atmospheric pressure.

33. The method according to claim 32, wherein the pressure of the gas is about 3 psi above atmospheric pressure.

FIG. 1

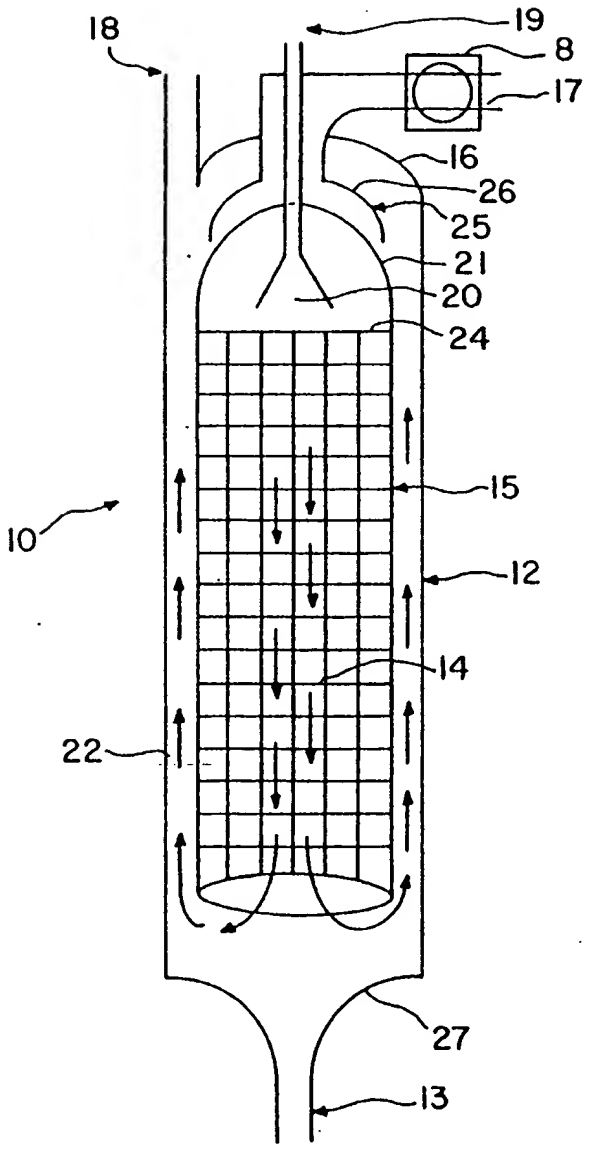


FIG.2

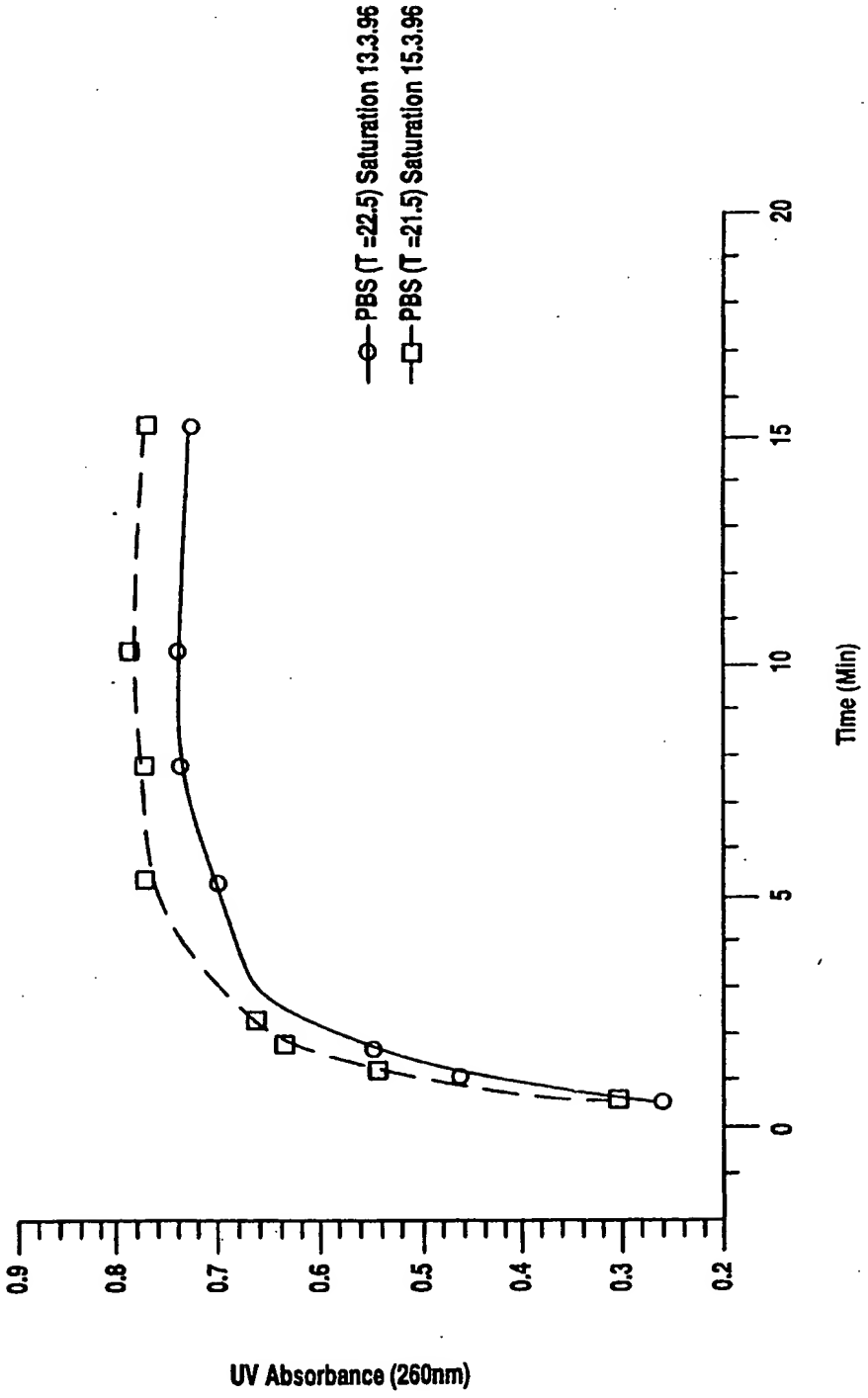

PBS (T =22.5) Saturation 13.3.96
PBS (T =21.5) Saturation 15.3.96
0
5
10
15
20
Time (Min)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
UV Absorbance (260nm)

FIG.3

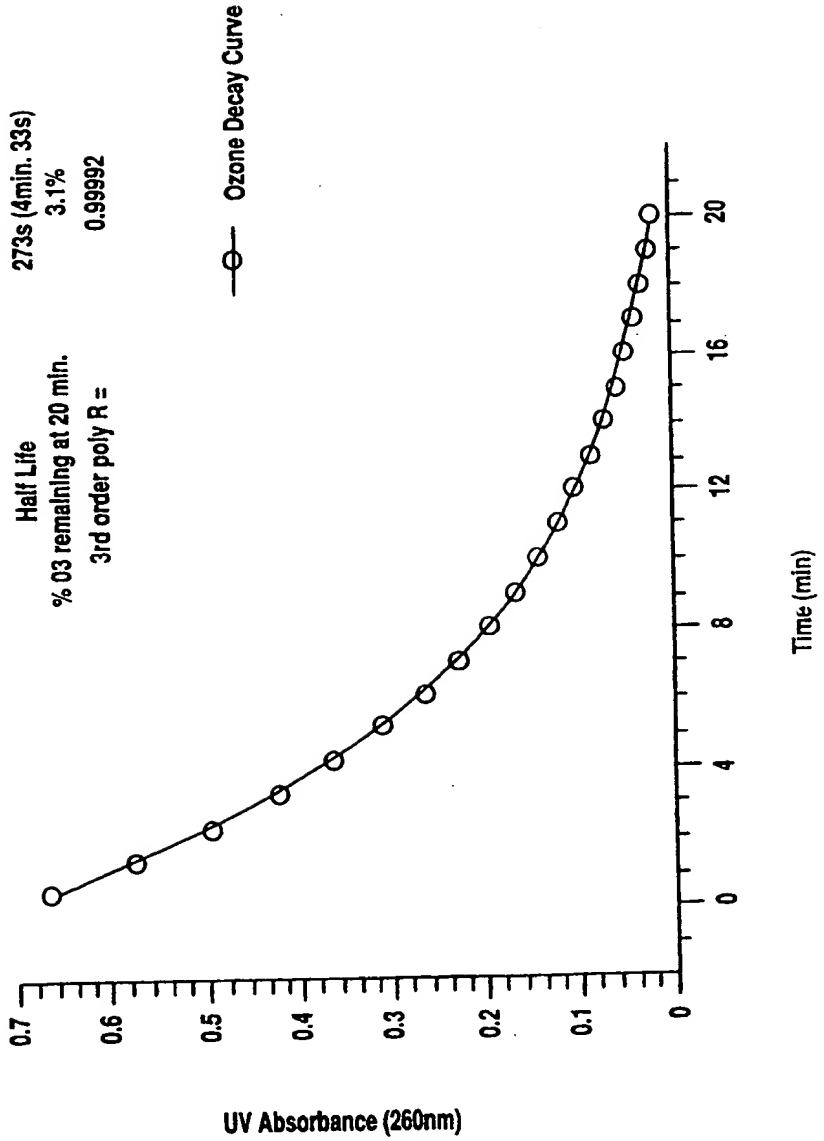

Ozone Decay Curve
Half Life 273s (4min. 33s)
% 03 remaining at 20 min. 3.1%
3rd order poly R = 0.99992
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
UV Absorbance (260nm)
0
4
8
12
16
20
Time (min)

FIG.4

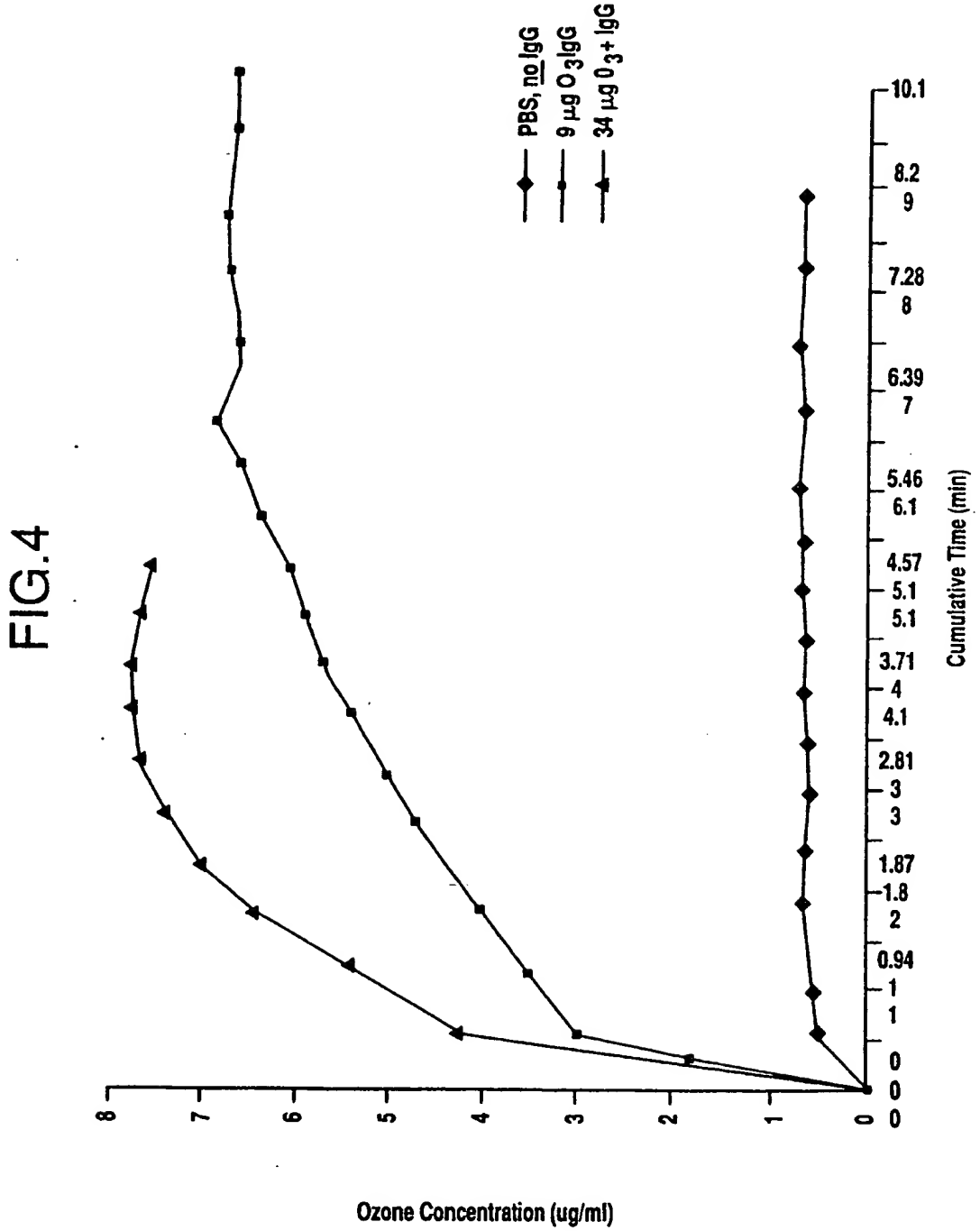

PBS, no IgG
9 µg O3IgG
34 µg O3+ IgG
10.1
8.2
9
7.28
8
6.39
7
5.46
6.1
4.57
5.1
5.1
3.71
4
4.1
2.81
3
3
1.87
1.8
2
0.94
1
1
0
0
0
Cumulative Time (min)
8
7
6
5
4
3
2
1
0
Ozone Concentration (ug/ml)

FIG.5

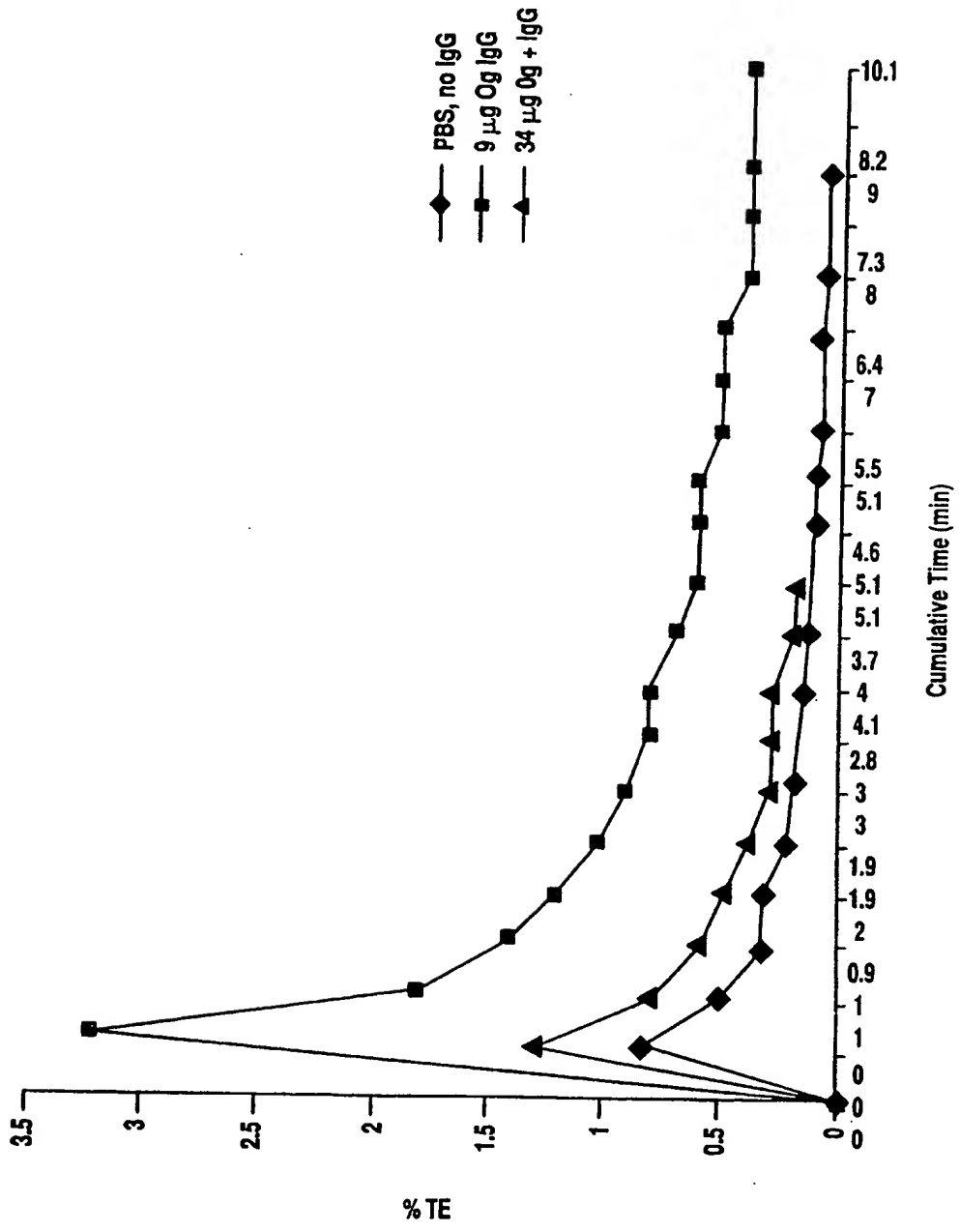

PBS, no IgG
9 µg Og IgG
34 µg Og + IgG
% TE
3.5
3
2.5
2
1.5
1
0.5
0
Cumulative Time (min)
10.1
8.2
9
7.3
8
6.4
7
5.5
5.1
4.6
5.1
5.1
3.7
4
4.1
2.8
3
3
1.9
1.9
2
0.9
1
1
0
0
0

FIG.6

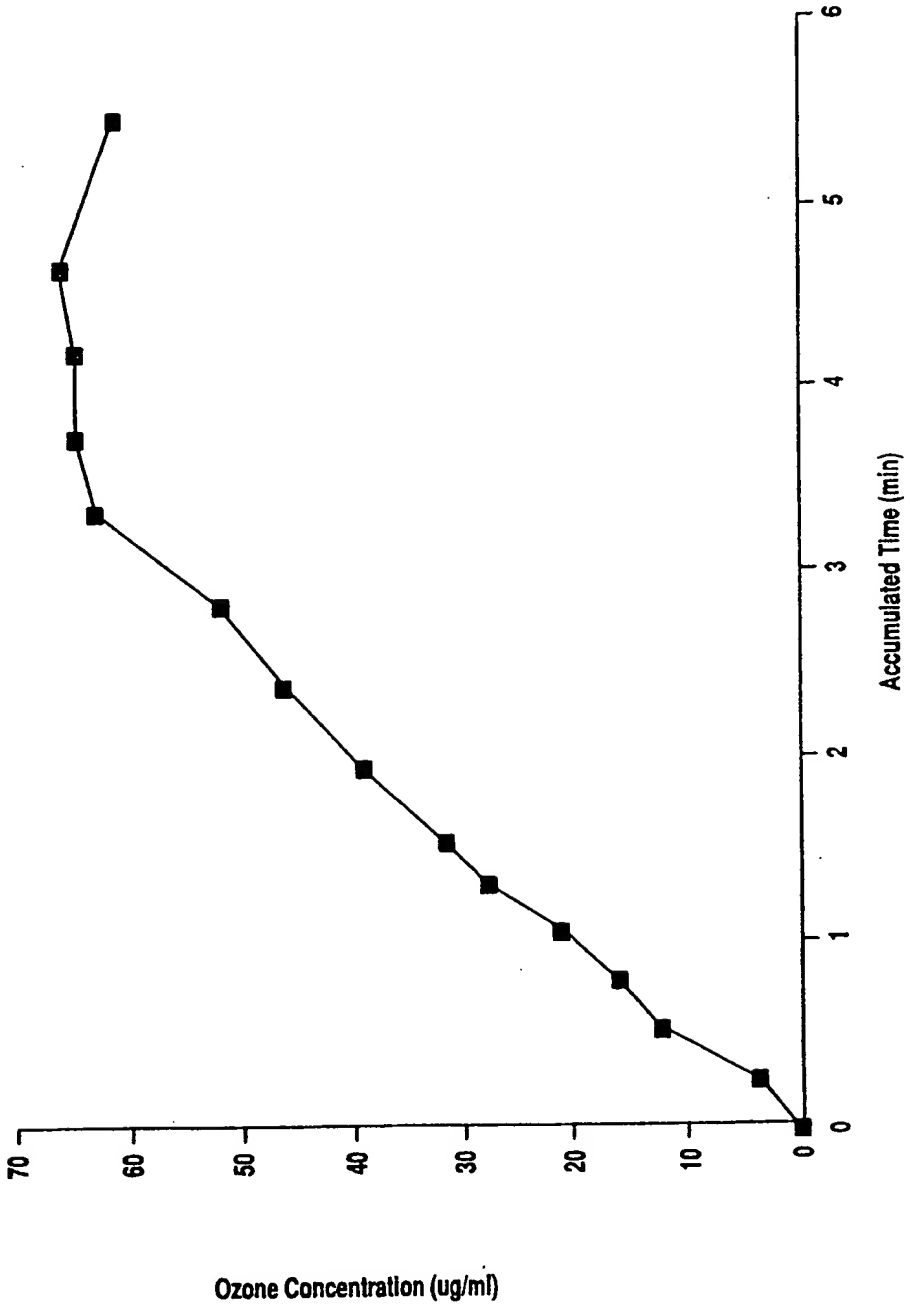

Accumulated Time (min)
0
1
2
3
4
5
6
Ozone Concentration (ug/ml)
0
10
20
30
40
50
60
70

FIG.7

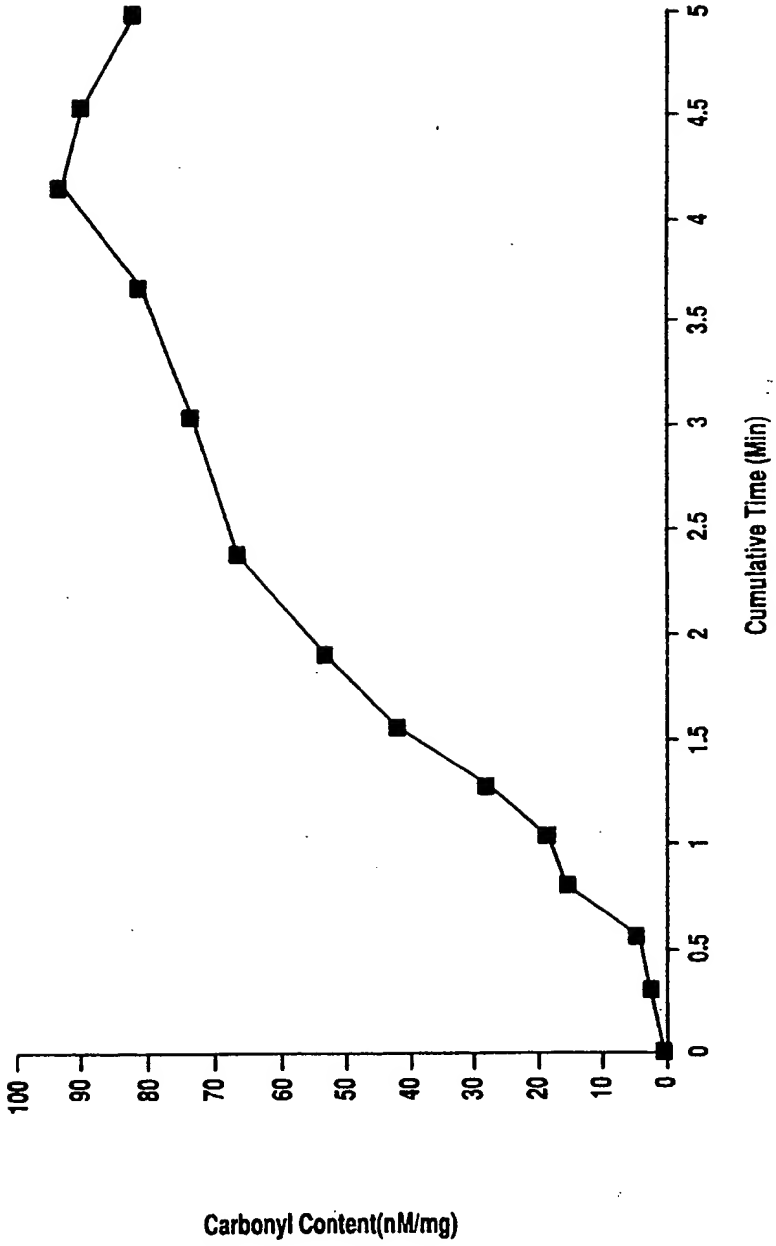

Cumulative Time (Min)
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
Carbonyl Content(nM/mg)
100
90
80
70
60
50
40
30
20
10
0

FIG.8

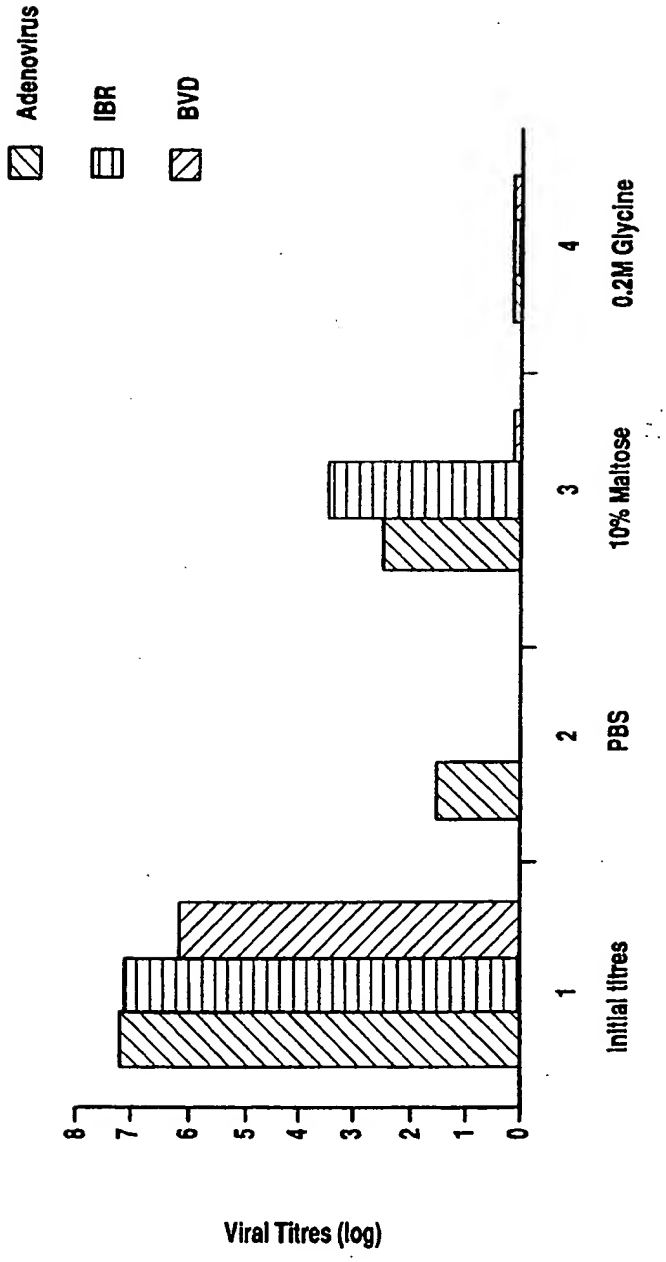

Adenovirus
IBR
BVD
8
7
6
5
4
3
2
1
0
Viral Titres (log)
1
Initial titres
2
PBS
3
10% Maltose
4
0.2M Glycine

FIG.9A

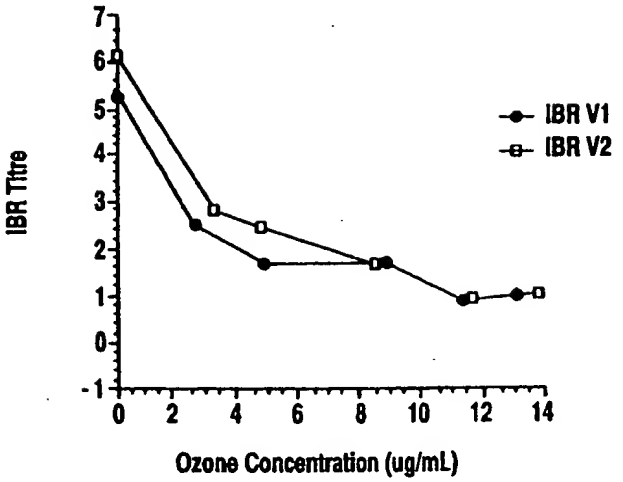


FIG.9B

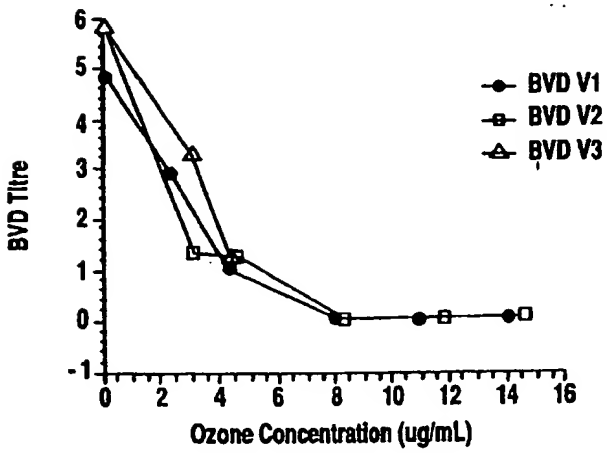


FIG.9C

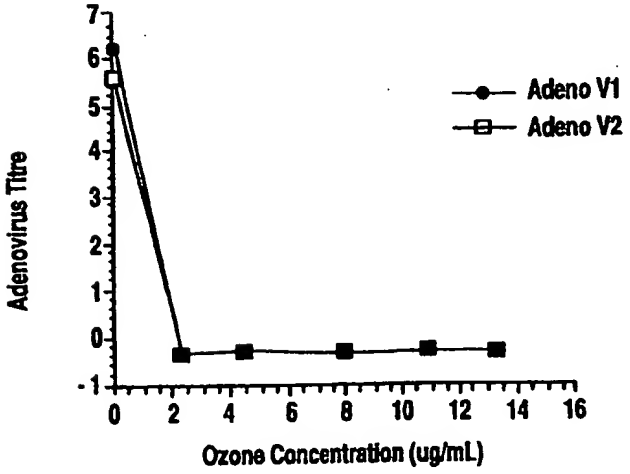


FIG.10C

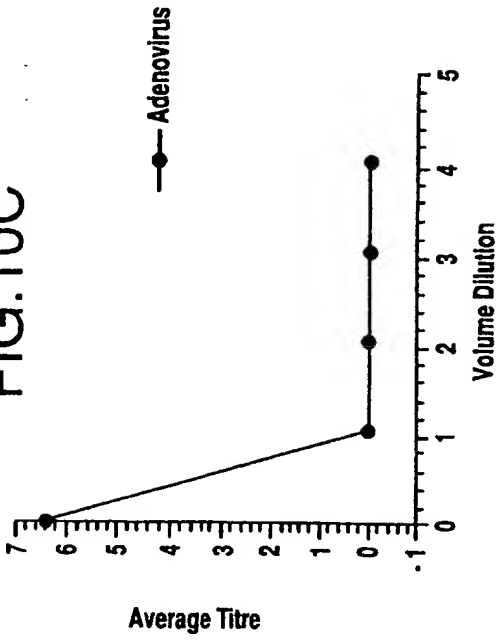


FIG.10D

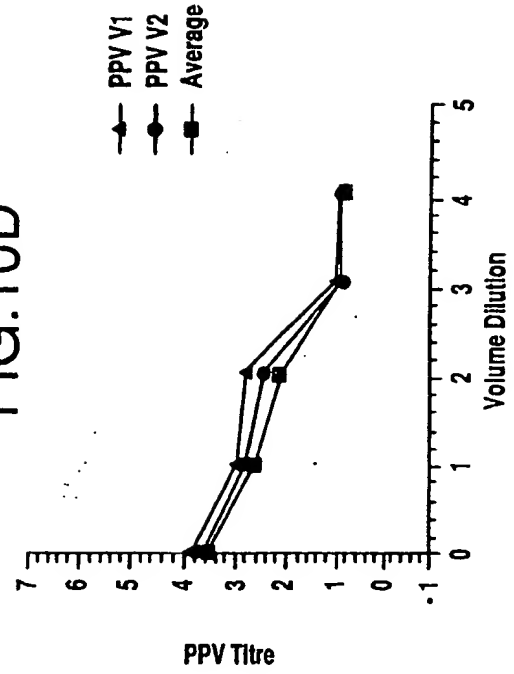


FIG.10A

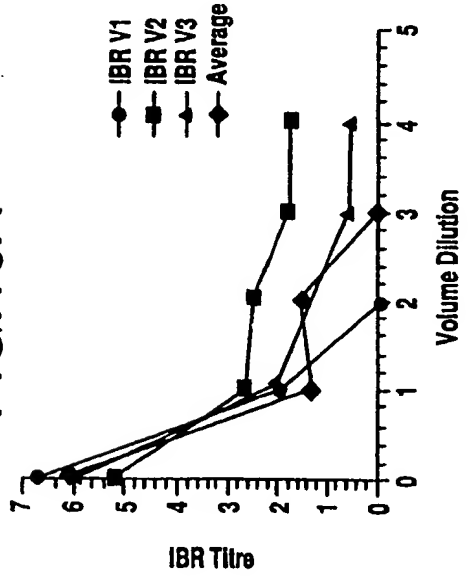


FIG.10B

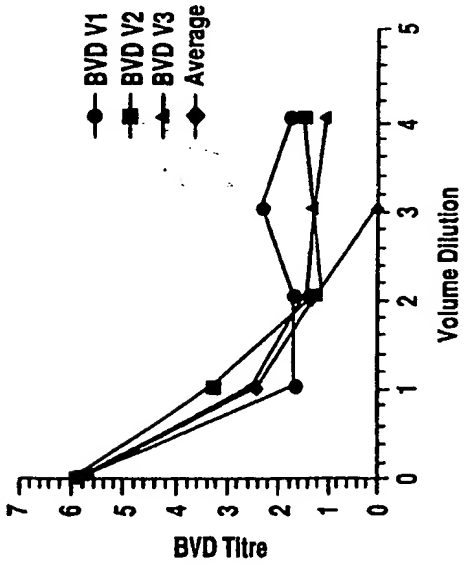


FIG.11A

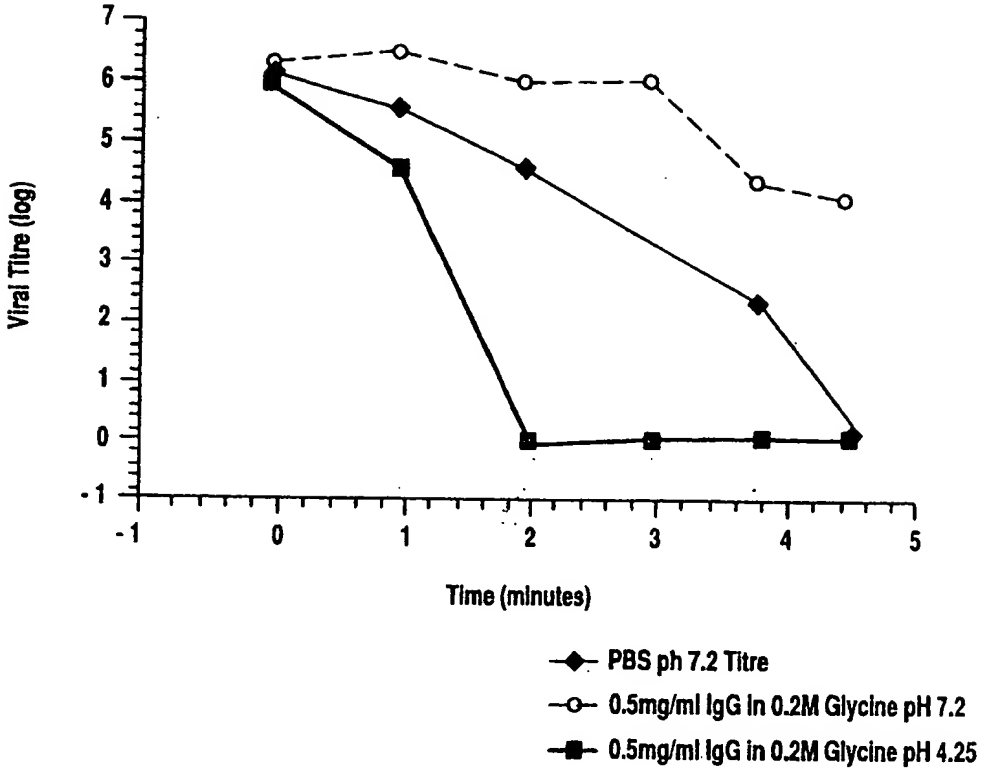


FIG.11B

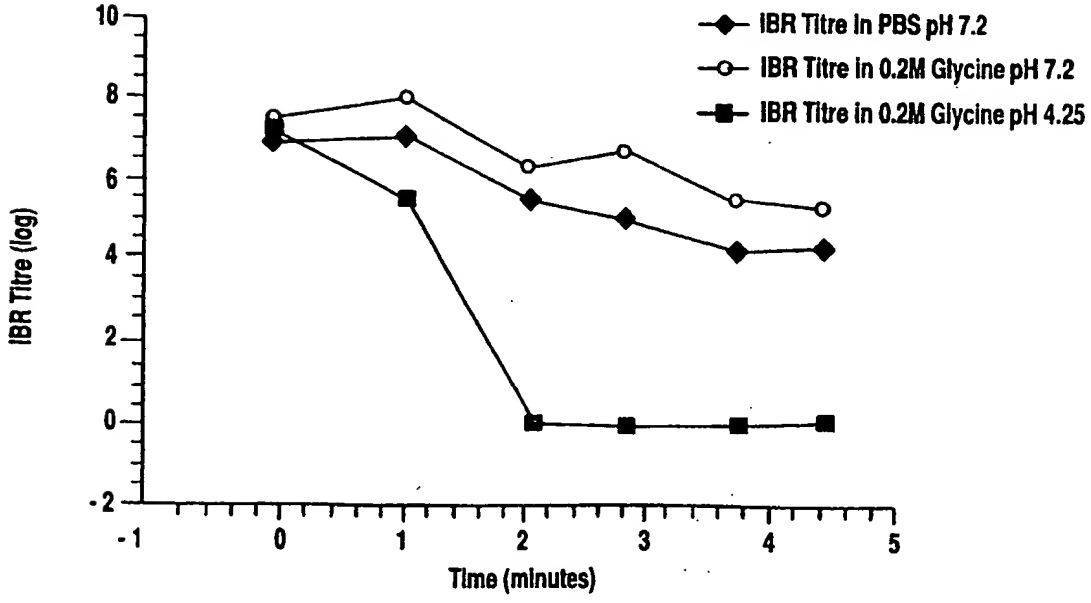
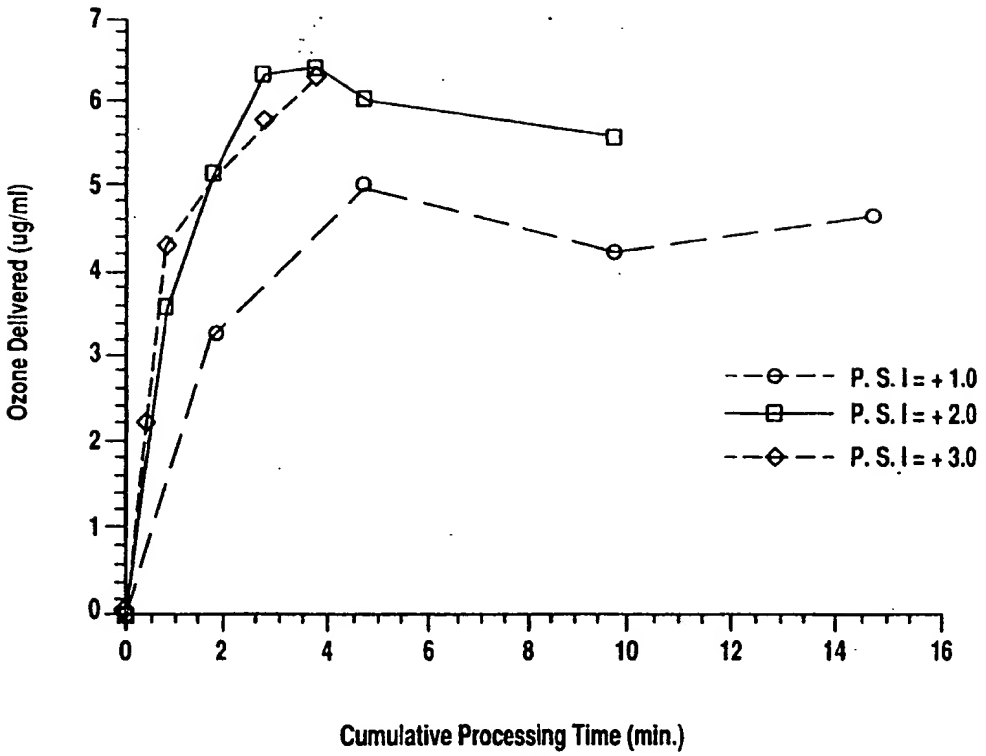


FIG.12


Ozone Delivered (ug/ml)
0
1
2
3
4
5
6
7
0
2
4
6
8
10
12
14
16
Cumulative Processing Time (min.)
P. S. I = + 1.0
P. S. I = + 2.0
P. S. I = + 3.0

INTERNATIONAL SEARCH REPORT

Inte. ional Application No
PCT/IB 97/01242

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61L2/20

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DATABASE WPI Section Ch, Week 9526 Derwent Publications Ltd., London, GB; Class B07, AN 95-194730 XP002052341 & CN 1 084 769 A (XING J) , 6 April 1994 see abstract	1-33
A	PATENT ABSTRACTS OF JAPAN vol. 017, no. 533 (C-1114), 27 September 1993 & JP 05 148114 A (TAKEDA CHEM IND LTD), 15 June 1993, see abstract	1-33
A	EP 0 535 612 A (OLYMPUS OPTICAL CO) 7 April 1993 see claims; examples 1-8	1-33

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of theinternational search	Date of mailing of the international search report
16 January 1998	28/01/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	ESPINOSA, M

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INTERNATIONAL SEARCH REPORT

Inte· ɔnal Application No
PCT/IB 97/01242

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	EP 0 261 032 A (ASS REGIO TRANSFUSION SANGUINE) 23 March 1988 see the whole document	1-33
A	US 4 640 782 A (BURLESON JAMES C) 3 February 1987 see claims	
A	EP 0 199 479 A (IMMUNOLOGICS) 29 October 1986 see claims & US 4 632 980 A cited in the application	1-33

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INTERNATIONAL SEARCH REPORT

information on patent family members

Inte onal Application No
PCT/IB 97/01242

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EP 0261032 A	23-03-88	FR 2603805 A	18-03-88
		JP 63123377 A	27-05-88
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EP 0199479 A	29-10-86	US 4632980 A	30-12-86
		CA 1269935 A	05-06-90
		HK 45193 A	14-05-93
		JP 1784817 C	31-08-93
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